Cleavage of the Carboxyl-Terminus of LEACS2, a Tomato 1-Aminocyclopropane-1-Carboxylic Acid Synthase Isomer, by a 64-kDa Tomato Metalloprotease Produces a Truncated but Active Enzyme

Jian-Feng LI[1, 2], Robert QI[3], Liang-Hu QU[2], Autar K Mattoo[4] and Ning LI[1, 2*]

(1. *Department of Biology*, *The Hong Kong University of Science and Technology*, Hong Kong SAR, China;

2. *Key Laboratory of Gene Engineering of the Ministry of Education*, *Zhongshan University*, Guangzhou 510275, China;

3. *Department of Biochemistry*, *The Hong Kong University of Science and Technology*, Hong Kong SAR, China;

4. *Sustainable Agricultural Systems Laboratory*, *United States Department of Agriculture-Agriculture Research Service*,

Beltsville, Maryland 20705-2350, USA)

Abstract: 1-Aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) is the principal enzyme in phytohormone ethylene biosynthesis. Previous studies have shown that the hypervariable C-terminus of ACS is proteolytically processed *in vivo*. However, the protease responsible for this has not yet been identified. In the present study, we investigated the processing of the 55-kDa full-length tomato ACS (LeACS2) into 52-, 50- and 49-kDa truncated isoforms in ripening tomato (*Lycopersicon esculentum* Mill. cv. Cooperation 903) fruit using the sodium dodecyl sulfate-boiling method. Meanwhile, an LeACS2-processing protease was purified via multi-step column chromatography from tomato fruit. Subsequent biochemical analysis of the 64-kDa purified protease revealed that it is a metalloprotease active at multiple cleavage sites within the hypervariable C-terminus of LeACS2. N-terminal sequencing and matrix-assisted laser desorption/ionization time-of-flight analysis indicated that the LeACS2-processing metalloprotease cleaves at the C-terminal sites Lys^{438}, Glu^{447}, Lys^{448}, Asn^{456}, Ser^{460}, Ser^{462}, Lys^{463}, and Leu^{474}, but does not cleave the N-terminus of LeACS2. Four C-terminus-deleted (26–50 amino acids) LeACS2 fusion proteins were overproduced and subjected to proteolysis by this metalloprotease to identify the multiple cleavage sites located on the N-terminal side of the phosphorylation site Ser^{460}. The results indisputably confirmed the presence of cleavage sites within the region between the α-helix domain (H14) and Ser^{460} for this metalloprotease. Furthermore, the resulting C-terminally truncated LeACS2 isoforms were active enzymatically. Because this protease could produce LeACS2 isoforms *in vitro* similar to those detected *in vivo*, it is proposed that this metalloprotease may be involved in the proteolysis of LeACS2 *in vivo*.

Key words: 1-aminocyclopropane-1-carboxylic acid synthase; cleavage sites; metalloprotease; proteolytic processing.

Tomato fruit ripening is a complex process involving extensive structural, metabolic and physiological changes. Ethylene is a potent and volatile plant hormone that plays a central role in controlling the onset of ripening in tomato and other fruits (Gane 1934; Oeller *et al.* 1991; Alexander and Grierson 2002; Klee 2002).

1-Aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase are two key enzymes that mediate the critical committed steps in ethylene biosynthetic pathway from *S*-adenosylmethionine (AdoMet) to ACC and then to ethylene (Fluhr and Mattoo 1996; Bleecker and Kende 2000). However, the

rate-limiting step in ethylene biosynthesis is ACC formation by ACS (Kende 1993). The ACS is encoded by a multigene family in tomato and in many other plants (Rottmann *et al.* 1991; Nakatsuka *et al.* 1998) and recent molecular biology studies have already demonstrated that the transcription of distinct subsets of ACS genes can be induced by different developmental cues and environmental stresses (Ge *et al.* 2000). In the tomato fruit, the expression of two ACS genes, namely *LeACS2* and *LeACS4*, is induced by ripening signals in a concerted fashion (Nakatsuka *et al.* 1998), but the predominant transcript is *LeACS2* (van der Straeten *et al.* 1990; Oeller *et al.* 1991; Li *et al.* 1992; Nakatsuka *et al.* 1998). Although the post-transcriptional regulation of *LeACS* gene expression has not been studied extensively, the post-translational regulatory mechanisms of *LeACS* have been investigated in a number of studies (Satoh and Esashi 1986; Spanu *et al.* 1990; Felix *et al.* 1991, 1994; Kim and Yang 1992; Li and Mattoo 1994; Tatsuki and Mori 2001; Chae *et al.* 2003; Tsuchisaka and Theologis 2004; Wang *et al.* 2004)

Based on the findings of previous studies, ACS enzymes have been shown to be regulated at the post-translational level by four distinct pathways. The first pathway is a mechanism-based inhibition, in which the substrate, AdoMet, can inactivate ACS by forming a covalent link between its vinylglycine moiety and the lysine residue within the active site of the enzyme (Satoh and Esashi 1986). This mechanism-based inactivation has been shown to be rapid and to occur both *in vitro* and *in vivo* (Spanu *et al.* 1990; Kim and Yang 1992). A second post-translational regulatory mechanism involves heterodimeric interactions between subunits of ACS isomers (Tsuchisaka and Theologis 2004). Such heterodimerization has been proposed to enhance the diversity of the ACS family and to allow ACC production to proceed within a broad gradient of AdoMet concentrations in various plant cell types. The third post-translational regulatory mechanism for ACS is the phosphorylation of ACS, which was proposed to increase the stability of this enzyme, whereas dephosphorylation decreased it (Spanu *et al.* 1994). Indeed, later

studies on transgenic tobacco plants have found that a mitogen-activated protein kinase (MAPK)-dependent phospho-transfer pathway is involved in enhancement of ACS activity *in vivo* (Kim *et al.* 2003). The phosphorylation site on the tomato LeACS2 isozyme was later found to be Ser[460], which is located within the hypervariable C-terminal region, based on both *in vitro* and *in vivo* experiments (Tatsuki and Mori 2001), the Ser[460] residue of LeACS2 was postulated to be phosphorylated by calcium-dependent protein kinases (CDPKs; Sebastia *et al.* 2004). The fourth post-translational regulatory mechanism for ACS is the proteolytic modification of the C-terminus. Modification of *Arabidopsis* AtACS5 and AtACS9 by *eto2* and *eto3* mutations, respectively, were found to confer greater stability *in vivo* (Chae *et al.* 2003). Deletion of the C-terminus of tomato LeACS2 was found to have effects on the overall catalytic efficiency and structural confirmation of this enzyme (Li and Mattoo 1994). Further studies of an *eto1* mutant have also revealed that a substrate-specific adaptor protein, namely ethylene-overproducer 1 (ETO1), binds to the C-terminus of an ACS (AtACS5) in *Arabidopsis* (Wang *et al.* 2004) and its binding decreases the catalytic activity, as well as the stability, of AtACS5 *in vivo*. A model for C-terminal modification regulatory mechanism was also put forward for ACS (Wang *et al.* 2004).

In previous studies, some ACS isozymes prepared from plant tissues showed a smaller molecular mass than that deduced from their amino acid sequences and were also smaller than the *in vitro* translation product or the recombinant counterparts expressed in *Escherichia coli* (Nakajima *et al.* 1988; Sato and Theologis 1989; Edelman and Kende 1990; van der Straeten *et al.* 1990; Sato *et al.* 1991) and the proteolytic truncation of ACS may occur despite the presence of protease inhibitors during the purification procedure (Sato *et al.* 1991). In addition, Sato *et al.* (1991) have detected an *in vivo* truncated ACS in zucchini fruits using the sodium dodecyl sulfate (SDS)-boiling method. Furthermore, because ACS with a long hyperviable C-terminus has been proven to be phosphorylated at the

C-terminal region *in vivo* and *in vitro* (Tatsuki and Mori 2001), the failure to detect the phosphorylated ACS in tomato suspension-cultured cells by Spanu *et al.* (1994) may suggest that the C-terminus of ACS may be proteolytically cleaved off from the enzyme *in vivo*. Taken together, these studies suggest that the proteolysis of ACS is likely to occur *in vivo*.

One more necessary piece of evidence for addressing the *in vivo* proteolytic modification of ACS is to identify the ACS-processing proteases from plants. However, thus far no protease participating in the potential *in vivo* proteolysis of ACS has been isolated. In the present study, we observed the *in vivo* proteolysis of ACS (LeACS2) in ripe tomato fruits using the SDS-boiling method and a 64-kDa metalloprotease that could process LeACS2 *in vitro* was purified by column chromatography and found to be homologous to M41 metalloprotease by the electrospray ionisation mass spectrometry (ESI-MS) analysis. This tomato metalloprotease (TMP) could specifically cleave the C-terminus of LeACS2 at multiple sites on the N-terminal site of Ser^{460} to produce a truncated but active enzyme. The present study provides an entry point for further investigation of the potential proteolytic modification of ACS in plants.

1   Materials and Methods

1.1   Plant materials

All tomato (*Lycopersicon esculentum* Mill. cv. Co-operation 903) fruits used in the present study were gifts from Nanjing Botanical Garden Memorial SUN Yat-Sen, Nanjing, China.

1.2   Preparation of LeACS2 antibodies

C-terminally truncated LeACS2 was overexpressed in *Escherichia coli* transformed with the recombinant plasmid pETdel-1 (Li and Mattoo 1994). The *del-1* LeACS2 protein was then purified and emulsified with Freund's complete adjuvant (Sigma, St Louis, MO, USA). A rabbit was immunized twice intradermally with 200 μg protein and the LeACS2-specific antibodies were affinity purified using a HiTrap NHS-activated column (Pharmacia Biotech AB, Uppsala, Sweden) that had been pre-cross-linked with the *del-1* LeACS2 antigens.

1.3   Immunodetection of *in vivo* LeACS2 proteolysis

Intact fresh tomato fruit samples were frozen in liquid nitrogen and quickly smashed into small pieces and ground into frozen powders. Wounded tomato fruit powders were prepared by slicing the fruit into pieces and incubating them at room temperature for 12 h prior to grinding. Frozen powder (200 mg) was mixed rapidly with an equal volume (approximately 200 μL) of preboiled 2×SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer (120 mmol/L Tris-HCl, pH 6.8, 20% glycerol, 4% electrophoresis-grade SDS, 0.01% bromophenol blue, 0.2% β-mercaptoethanol) and boiled immediately at 100 °C for 10 min. Extracts were clarified by centrifugation at 10 000g for 15 min at room temperature and 20-μL aliquots of each supernatant were analyzed by SDS-PAGE, followed by immunoblotting with anti-LeACS2 serum as the primary antibodies and horseradish peroxidase (HRP)-conjugated antibodies against rabbit IgG as the secondary antibodies. The resulting signals were visualized by diaminobenzidine (DAB) chromogen (Sigma).

1.4   Construction of recombinant expression plasmids

Full-length LeACS2 cDNA was generated by reverse transcription-polymerase chain reaction (RT-PCR) using the primers ACSNF (5'-CGCATATGGGATTTGA-GATTGCAAAG-3'; the underlined section is an *Nde*I site) and ACSBR (5'-AAGGATCCACGAACTAATG-GTGAGGGAGGA-3'; the underlined section is a *Bam*HI site) and was digested with *Nde*I/*Bam*HI and inserted into the pET30a vector (Novagen) to produce pETACS2GST. A Factor Xa cleavage site was introduced into the border region between LeACS2 and its C-terminal glutathione *S*-transferase (GST) tag. All cDNA inserts were sequenced to confirm their identities. Recombinant plasmids carrying four C-terminal-deletion LeACS2 mutants (pETdel1GST–pETdel4GST) were constructed as follows: the 3' end of LeACS2 cDNA was replaced individually with four PCR fragments encoding the truncated LeACS2

C-terminus. These PCR products were predigested with *Eco*RI/*Bam*HI and then ligated to a linearized pETACS2GST plasmid that was predigested with the same pair of restriction endonucleases. The primers used for PCR amplification of the truncated 3' end of the LeACS2 cDNA were: ACSE(F), 5'-GCCTGAATTCAGAAAAGCGATTGC-3' (underlining indicates an *Eco*RI site); Del1(R), 5'-CGGGATCCAAGTCTCAAATTATTCTTCTTCC-3'; Del2(R), 5'-CGGGATCCTTTATCTCCACTTTTCTC-3'; Del3(R), 5'-CGGGATCCCTCAACACC-TACGAACCT-3'; and Del4(R), 5'-CGGGATCCTAC-GAACCTCCGAATCCT-3' (underlining indicates a *Bam*HI site).

1.5   Overexpression and purification of full-length and C-terminal-truncated LeACS2 proteins

Full-length and truncated LeACS2 proteins were expressed as C-terminal GST fusion products in a heterologous *E. coli* BL21 star™ (DE3) pLysS system (Invitrogen Corporation, Carlsbad, CA, USA). *E. coli* harboring the recombinant plasmids pETACS2GST and pETdel1GST–pETdel4GST were grown overnight at 37 °C in 3 mL LB medium, containing 20 µg/mL kanamycin and 25 µg/mL chloramphenicol, and were then diluted 100-fold with 2×YT medium, containing 20 µg/mL kanamycin, and grown at 37 °C until the OD_{600} of the culture reached 0.6. The overexpression of both full-length and truncated LeACS2 proteins was induced by 0.1 mmol/L IPTG at 30 °C for 5 h. Cells were then harvested by centrifugation at 8 000g for 5 min and, following a wash with 20 mL extraction buffer (40 mmol/L Tris-HCl, 20 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L dithiothreitol (DTT), pH 7.8), the pellets were resuspended in 6 mL extraction buffer and sonicated on ice. Cell lysates were incubated with 6 µL Benzonase (Novagen) at 37 °C for 10 min and centrifuged at 18 000g for 20 min at 4 °C and the supernatant was subjected to a GST affinity column (Novagen) that had been pre-equilibrated with phosphate-buffered saline (PBS). The column was then washed with 30 bed volumes of PBS and eluted with two bed volumes of glutathione elution buffer (10 mmol/L reduced glutathione,

50 mmol/L Tris-HCl, pH 8.0).

1.6   Assay for proteolytic activity

The proteolysis reaction was performed in a buffer, containing 20 mmol/L Tris-HCl, 100 mmol/L NaCl, 2 mmol/L $CaCl_2$, 5 µmol/L $ZnCl_2$, pH 8.0, in which LeACS2-GST fusion proteins were used as substrates. Either crude protease extract or purified TMP was added to the proteolysis reaction buffer and the reactions proceeded for 3–6 h at 37 °C. In partial digestion experiments, the proteolytic reactions were terminated between 0.2 and 6.0 h and the proteolytic products were analyzed immediately by SDS-PAGE and immunoblotting.

1.7   Purification of TMP from tomato

Details regarding the purification of TMP from tomato have been published elsewhere (Li *et al.* 2005).

1.8   Determination of the TMP cleavage site

The completely processed LeACS2-GST fusion proteins by TMP were subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric analysis according to the methods described by Janzik *et al.* (2000). The mass spectra of small peptides (with molecular weights less than 10 kDa) were obtained with an automated mode using a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Voyager-DE-STR; Applied Biosystems, Scoresby, Australia). The spectra were annotated automatically using PSI software (Applied Biosystems). Cleavage sites were deduced from the peptide sequences identified. In addition, the partially processed LeACS2 fusion proteins by TMP were resolved by SDS-PAGE and both the N-terminal region of LeACS2 (approximately 49 kDa) and the short stretches of the C-terminal regions of LeACS2 fused to GST (approximately 29 kDa), were subjected to N-terminal sequencing according to the method of Edman (1950), which was performed at the Proteomics Center of the Shanghai Genecore Company (Shanghai, China).

1.9   1-Aminocyclopropane-1-carboxylic acid synthase activity assay

The ACS samples were assayed for enzymatic

activities as described previously (Li *et al.* 1996) and the quantities of ACC formed in each reaction mix were determined according to the method of Lizada and Yang (1979).

2   Results

2.1   Detection of *in vivo* proteolysis of LeACS2

In the present study, we examined the *in vivo* profile of LeACS2 in unwounded or wounded ripe tomato fruits using the previously reported SDS-boiling method (Sato *et al.* 1991; Tatsuki and Mori 2001). In the case of unwounded ripe tomato fruits, immunoblotting with anti-LeACS2 polyclonal antibodies clearly showed that both the full-length and truncated forms of this enzyme (ACS2Δ^1, ACS2Δ^2, and ACS2Δ^3) existed *in vivo* with molecular weights of 55, 52, 50, and 49 kDa, respectively (Fig. 1). The predominant isoform of LeACS2 appeared to be the truncated 49-kDa ACS2Δ^3 species, whereas the ACS2Δ^1 and ACS2Δ^2 isoforms appeared to be minor truncation intermediates. In contrast, in wounded ripe fruit tissues, the 55-kDa
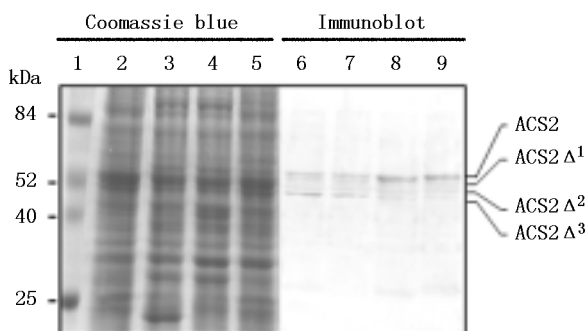

Fig. 1.   *In vivo* and *in vitro* proteolysis of LeACS2. Total cellular proteins were extracted from intact or wounded tomato fruits using the sodium dodecyl sulfate (SDS)-boiling method and analyzed by SDS-polyacrylamide gel electrophoresis. Lane 1, prestained protein molecular weight markers; lane 2, pink fruit; lane 3, red fruit; lane 4, wounded pink fruit; lane 5, wounded red fruit. Immunoblots of protein samples were probed with polyclonal anti-LeACS2 antibodies; lane 6, pink fruit; lane 7, red fruit; lane 8, wounded pink fruit; lane 9, wounded red fruit. The full-length LeACS2 protein and *in vivo* proteolytically truncated LeACS2 isomers are designated as ACS2, ACS2Δ^1, ACS2Δ^2, and ACS2Δ^3, respectively.

full-length LeACS2 was predominant (Fig. 1), which is in agreement with previous findings (Tatsuki and Mori 2001). Based on our initial observations of LeACS2 isoforms in ripened fruit tissues, we reasoned that there may be a protease responsible for the proteolytic processing of this enzyme in ripe tomato fruits.

2.2   Purification of the protease responsible for the proteolytic processing of LeACS2 from tomato

To identify this protease, we first established an *in vitro* assay system using a LeACS2-GST hybrid protein as a substrate. The 55-kDa full-length LeACS2 protein was linked at its C-terminus to GST to facilitate the purification of recombinant substrate and to mark the difference between processed and unprocessed LeACS2 in the *in vitro* assays. To isolate the LeACS2-processing protease from tomato fruit, we used, in turn, DEAE, gel filtration, and MonoQ chromatography. Details of the purification procedure have been described previously (Li *et al.* 2005). The protein profiles of the protease extracts obtained from each purification step were fractionated on SDS-PAGE and visualized by Coomassie blue staining (Fig. 2a). It is evident that two protein species of distinct molecular weights, namely 64 and 50 kDa, were eventually purified following MonoQ chromatography (Fig. 2a). In addition, the levels of the 64-kDa species were steadily enriched 20-fold as the purification progressed, whereas the intensity of 50-kDa band was found to decrease following MonoQ chromatography. The final level of the 64-kDa protein was nearly 10-fold greater than that of the 50-kDa species. Hence, we postulated that the 64-kDa protein was the putative protease responsible for the proteolytic processing of LeACS2. This conclusion was further confirmed by ESI-MS analysis, in which the MS/MS fragmental mass spectra of the 50-kDa protein matched with tomato β-fructofuranosidase, whereas those of the 64-kDa protein were found to match with *Arabidopsis* T6H22.2 protein (GenBank accession no. NP_564711). This *Arabidopsis* homolog of TMP contains a highly conserved metalloprotease M41 domain shared by numerous M41 metalloproteases from different organisms
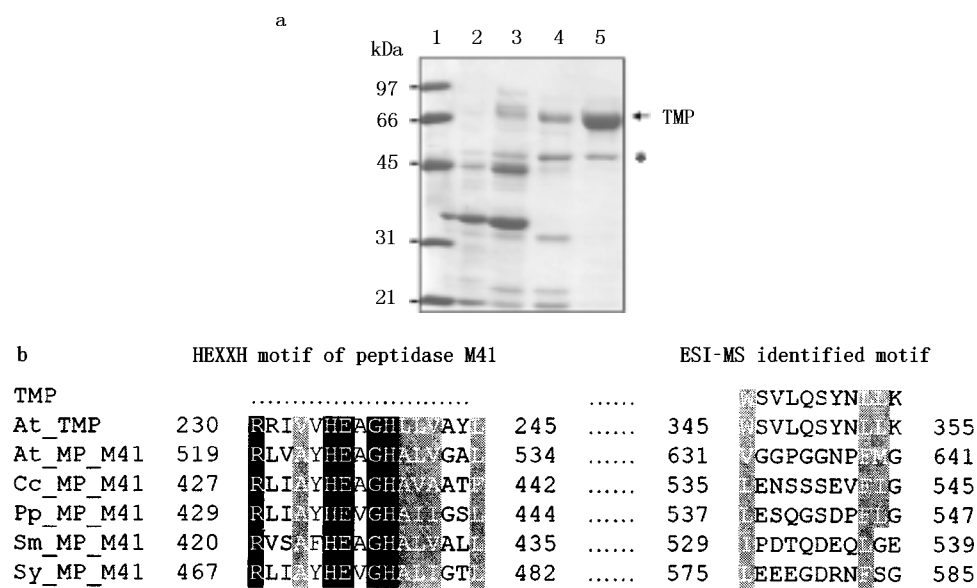
Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the protein profiles of each purification step. **a**. Lane 1, protein molecular weight markers. Crude protease extract (lane 2), DEAE fraction (lane 3), gel filtration fraction (lane 4), and MonoQ fraction (lane 5) were resolved on a 10% polyacrylamide gel followed by Coomassie brilliant blue staining. Each lane was loaded with 2 µg protein. The 64-kDa tomato protease is designated as tomato metalloprotease (TMP). The asterisk indicates the homolog of β-fructofuranosidase. **b**. Amino acid sequence alignment of T6H22.2 protein (the electrospray ionisation mass spectrometry (ESI-MS) identified homolog of TMP) and various M41 metalloproteases. At_TMP (accession no. NP_564711) denotes T6H22.2 protein in *Arabidopsis thaliana*. At_MP_M41 (accession no. Q39102), Cc_MP_M41 (accession no. O19922), Pp_MP_M41 (accession no. P51327), Sm_MP_M41 (accession no. P46508), and Sy_MP_M41 (accession no. P73179) are known M41 metalloproteases from *Arabidopsis thaliana*, *Cyanidium caldarium*, *Porphyra purpurea*, *Schistosoma mansoni*, and *Synechocystis* sp. PCC 6803, respectively. The invariant amino acid residues are labeled with black boxes and the highly conserved residues are labeled with gray boxes.

(Fig. 2b).

2.3 Determination of the cleavage sites on LeACS2

To determine the cleavage sites on LeACS2, a partial digestion of the fusion protein with the purified TMP was first performed. Figure 3a shows the time-course of TMP-mediated proteolysis of LeACS-GST *in vitro*. After 6 h incubation with purified TMP, the 83-kDa fusion protein was gradually processed into a 49-kDa truncated isoform, which was visualized by immunoblotting (Fig. 3a). It appeared that both the crude protease extract and purified TMP could generate the same 49-kDa truncated LeACS2 product and this *in vitro*-truncated LeACS2 species had a similar molecular size to *in vivo*-processed LeACS2 (ACS2Δ[3]; Fig. 3a). Further SDS-PAGE analysis of the partially

TMP-digested products showed that more than two bands were evident (Fig. 3b), indicating that at least two putative ACS2Δ polypeptides, 50- and 49-kDa products, were produced *in vitro*. However, the 49-kDa ACS2Δ species was the predominant form. In addition, two GST derivatives, namely GST1 (30-kDa) and GST2 (29-kDa), were also detectable (Fig. 3b). When the abundant 49-kDa ACS2Δ product and the GST1 and GST2 species were sequenced from the N-terminus, the first five amino acids were found to be Gly-Phe-Glu-Ile-Ala, Arg-Met-Tyr-Asp-Glu, and Ser-Ser-Pro-Ile-Pro, respectively. The first pentapeptide sequence of the 49-kDa protein was identical to the expected N-terminus of LeACS2 after excision of the N-terminal methionine (Hirel *et al.* 1989), suggesting that the N-terminus of LeACS2 was not cleaved by
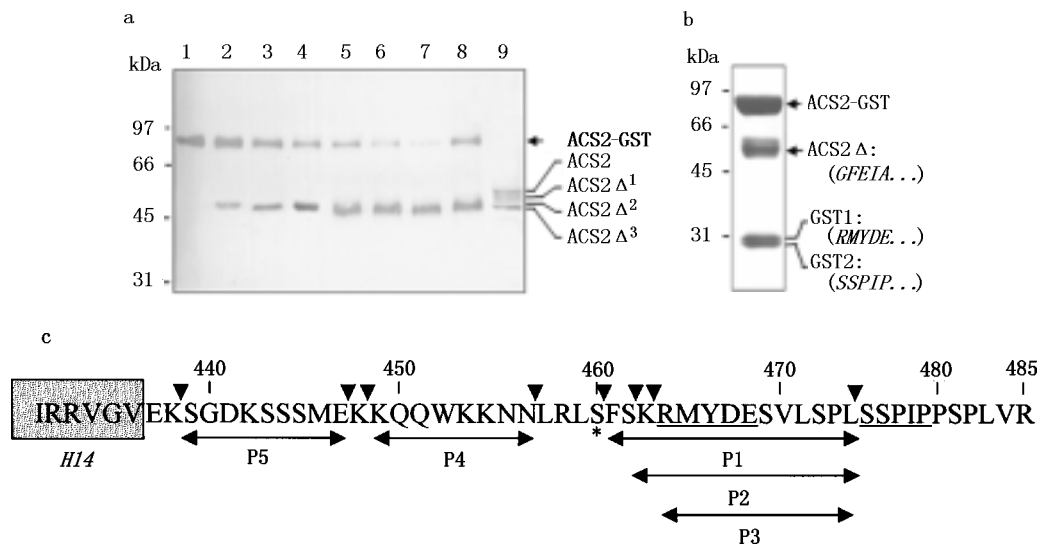
Fig. 3. Determination of the cleavage sites at the C-terminus of LeACS2. **a**. Time-course of LeACS2-glutathione *S*-transferase (GST) (0.5 µg) proteolysis by purified tomato metalloprotease (TMP; 1 ng). The proteolytic reaction was terminated at 0 h (lane 1), 0.2 h (lane 2), 0.5 h (lane 3), 1 h (lane 4), 1.5 h (lane 5), 3 h (lane 6), and 6 h (lane 7). Lane 8, proteolysis of LeACS2-GST with crude protease extract from red tomato fruit for 1.5 h. Total tomato protein preparations (lane 9) were extracted from pink tomato fruit tissues using the sodium dodecyl sulfate (SDS)-boiling method and resolved by SDS-polyacrylamide gel electrophoresis. Immunoblotting of these cleaved LeACS2 isomers was performed with anti-LeACS2 polyclonal antibodies. The fusion protein LeACS2-GST, the full-length LeACS2 protein, and the C-terminally truncated LeACS2 intermediates are indicated on the right. **b**. Partially digested LeACS2-GST fusion proteins (5 µg) by TMP (10 ng) were resolved on a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane followed by Coomassie brilliant blue staining. Both the N-terminal region of LeACS2 (ACS2Δ) and the C-terminal segments of LeACS2 that were linked to GST (GST1 and GST2) were N-terminally sequenced. The first five amino acids of these proteolytic products are shown in italic letters in parentheses. **c**. A matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric profile of proteolytically produced small peptides from the C-terminus of LeACS2. The LeACS2-GST fusion proteins (5 µg) were digested thoroughly with excess quantities (100 ng) of TMP. The TMP-processed C-terminal peptides of LeACS2, P1–P5, are delimited by horizontal arrows. The phosphorylation site, Ser[460], of LeACS2 is indicated by an asterisk. The N-termini of GST1 and GST2 are underlined. The vertical arrows indicate the TMP-mediated cleavage sites determined by N-terminal sequencing and MALDI-TOF analysis. The gray box, labeled *H14*, indicates the α-helix structure located at the C-terminal region of LeACS2.

Table 1 Proteolytic peptides of LeACS2 identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

| Peptide no.	Mass of the peak on MALDI-TOF (*m/z*)	Sequence	Predicted mass (Da)	Cleavage site on LeACS2	
				Start site	End site
P1	1 671.48	FSKRMYDESVLSPL	1 672	F[461]	L[474]
P2	1 437.44	KRMYDESVLSPL	1 438	K[463]	L[474]
P3	1 309.37	RMYDESVLSPL	1 309	R[464]	L[474]
P4	1 073.39	KQQWKKNN	1 073	K[449]	N[456]
P5	928.24	SGDKSSSME	927	S[439]	E[447]

Proteolytic peptides of LeACS2 obtained by complete tomato metalloprotease-mediated proteolysis were subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis. Cleavage sites were deduced from the peptide sequences identified.

TMP. Significantly, the N-terminal pentapeptide sequences of the 30- and 29-kDa proteins were identical to the C-terminal sequences of LeACS2 (Fig. 3b, c), indicating that TMP cleaves at the carboxyl side of Lys[463] and Leu[474], respectively, to generate 30- and 29-kDa proteolytic products.

It is of note that the total size of the two major LeACS-GST proteolytic products is 5 kDa less than the total molecular weight of the substrate (83 kDa), with no other proteolytic products detectable by 15% SDS-PAGE (data not shown). Therefore, we speculated that some extremely short peptides may have been produced by TMP digestion that may have been undetectable by SDS-PAGE. To determine whether this was the case, we performed MALDI-TOF mass spectrometric analysis on the completely TMP-digested products of LeACS2-GST. Five peptides with molecular masses of 1 671.475 1, 1 437.436 7, 1 309.369 7,

1 073.386 7 and 928.240 5 *m/z*, respectively, were detected to match five short regions, P1–P5, of the LeACS2 protein with identical molecular masses (Fig. 3c; Table 1). The presence of P3 further confirmed the TMP-mediated cleavage sites Lys[463] and Leu[474], which were first identified by N-terminal amino acid sequencing of GST1 and GST2 (Fig. 3b). Meanwhile, the identification of other short proteolytic peptides suggested that TMP also mediated multiple cleavages on LeACS2 at residue positions Lys[438], Glu[447], Lys[448], Asn[456], Ser[460], and Ser[462].

To determine whether any of these TMP-mediated cleavage sites were located on the N-terminal side of the Ser[460] phosphorylation site, we constructed a series of C-terminally truncated LeACS2 mutants, which lacked 26–50 amino acids from the wild-type C-terminus, and fused them to the GST tag (Del1-GST to Del4-GST; Fig. 4a). The molecular weights of these
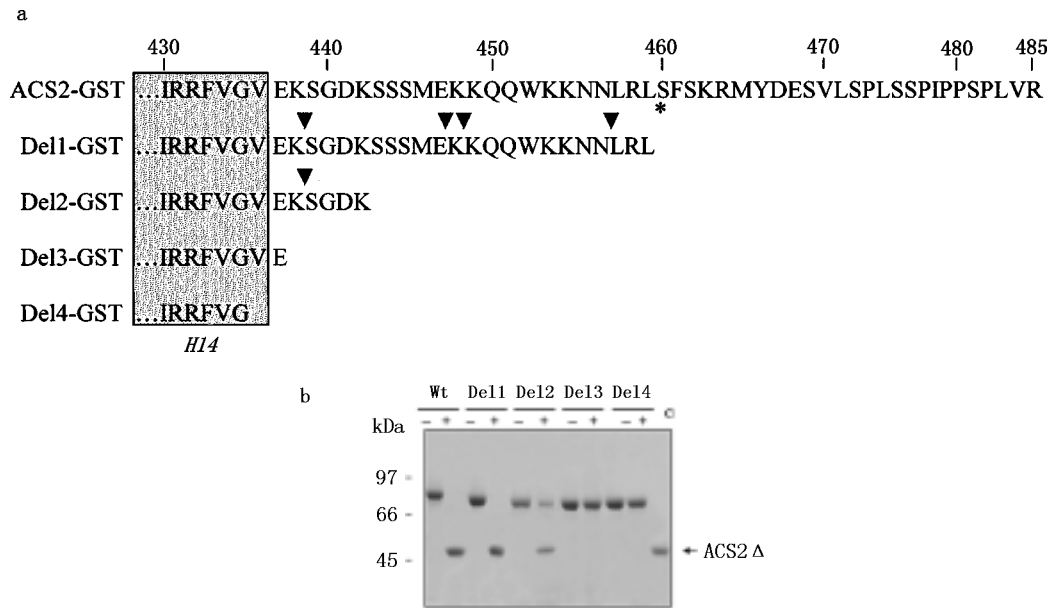

Fig. 4. Tomato metalloprotease (TMP)-mediated proteolysis of full-length and C-terminus-deleted LeACS2 proteins. **a**. Schematic diagram of the nested LeACS2 C-terminal deletions. Fusion proteins, containing full-length and C-terminus-deleted LeACS2, are denoted as ACS2-glutathione *S*-transferase (GST) and Del1-GST–Del4-GST, respectively. The hatched bar, labeled with *H14*, indicates the α-helix structure located at the C-terminal region of LeACS2. Arrows indicate the identified TMP cleavage sites. The phosphorylation site, Ser[460], of LeACS2 is marked by an asterisk. **b**. Proteolysis of full-length and C-terminus-deleted LeACS2 by TMP. Fusion proteins, ACS2-GST (wt), Del1-GST (Del1), Del2-GST (Del2), Del3-GST (Del3), and Del4-GST (Del4), were treated with (+) or without (–) excessive amounts of purified TMP. Lane C, LeACS2-GST fusion protein, serving as a control, digested with both Factor Xa and purified TMP. In each of these reactions, 1–1.5 μg fusion protein substrate was added.

fusion protein mutants were determined by SDS-PAGE to be 80, 78, 77, and 77 kDa, respectively (Fig. 4b). Proteolytic analysis of these mutants using purified TMP indicated that Del1-GST and Del2-GST were cleaved by TMP, whereas Del3-GST and Del4-GST were not (Fig. 4b). These studies confirm the presence of multiple TMP cleavage sites on the N-terminal side of Ser[460], implying that the putative regulatory C-terminal phosphorylation site would be removed from LeACS2 by this protease *in vitro*.

2.4 1-Aminocyclopropane-1-carboxylic acid synthase enzymatic activity assay for proteolytically modified LeACS2

In relation to the already documented post-translational regulatory mechanisms, one of the central questions raised by our findings is whether proteolytically modified LeACS2 retains its activity. To elucidate this, we purified the 49-kDa ACS2Δ and full-length LeACS2 products from TMP- and Factor Xa-mediated proteolysis of LeACS2-GST, respectively, and performed a separate ACS activity assay on each. At a *S*-adenosylmethionine concentration of 50 μmol/L, it was found that full-length and truncated enzymes had specific
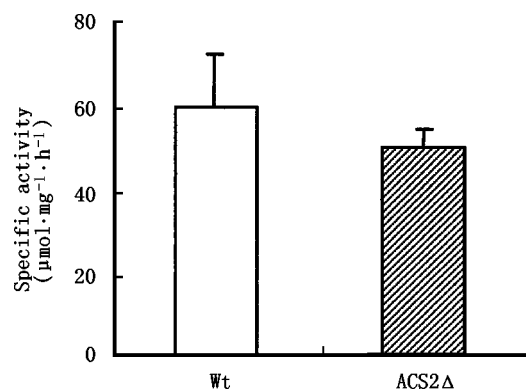
Fig. 5. Determination of activities of the full-length and tomato metalloprotease (TMP)-cleaved LeACS2. A hundred ng of the full-length (open bar, labeled with Wt) or C-terminus-cleaved (hatched bar, labeled with ACS2Δ) 1-aminocyclopropane-1-carboxylic acid (ACC) synthase was assayed respectively for the production of ACC. The concentration of substrate *S*-adenosylmethionine was 50 μmol/L. Error bars represent the standard errors of five replicates. One unit of ACC synthase activity is defined as 1 μmol of ACC formed per hour at 30 °C.

activities of 59.8 and 49.8 μmol·mg^{-1}·h^{-1}, respectively (Fig. 5), indicating that the TMP-processed ACS2Δ isomers are active enzymes.

3 Discussion

It has been documented previously that proteolytic modification of ACS may occur *in vivo* (Nakajima *et al.* 1988; Sato and Theologis 1989; Edelman and Kende 1990; van der Straeten *et al.* 1990; Sato *et al.* 1991). The successful detection *in vivo* of full-length ACS has relied largely on heat inactivation or the presence of proteinase inhibitors during protein sample preparation. When the ripe fruit extracts used in these analyses were heat-inactivated by SDS-boiling prior to being fractionated by SDS-PAGE, our Western blot experiments showed that the ripening-induced tomato ACS, LeACS2, exists as multiple molecular sizes (i.e. full-length (55-kDa) and truncated (52, 50 and 49-kDa) proteins); however, the wounding-induced LeACS2 exists mainly as the full-length form, indicating that some protease(s) may function during fruit ripening that may be disabled by wounding. However, the mechanisms underlying these observations are, as yet, unknown.

The principal reason why our immunoblotting experiments could detect the *in vivo* truncated isoforms of LeACS2 from ripe fruit was because the polyclonal antibodies were raised against a C-terminal truncated LeACS2 mutant (*del1*; Li and Mattoo 1994) and they have no preference for full-length LeACS2, which includes a peripheral (Huai *et al.* 2001) and antigenic C-terminus. Hence, the levels of four LeACS2 isozymes (55, 52, 50 and 49 kDa) may represent the steady state expression profile of LeACS2 at a given stage of tomato fruit ripening. Moreover, according to the results obtained in the present study as well as in other studies (Tatsuki and Mori 2001), it is conceivable that the proteolytic processing of LeACS2 can occur both *in vitro* and *in vivo*, as suggested previously (Kende 1993).

The multiple TMP cleavage sites within the C-terminal region of LeACS2 were determined primarily by MALDI-TOF analysis and N-terminal sequencing. The

P3 peptide predominated among the five short C-terminal peptides detected and, together with the N-terminal sequencing results of the proteolytic products GST1 and GST2 (Fig. 3b), we speculate that Lys[463] and Leu[474] are the primary cleavage sites for TMP and may produce the 52-kDa LeACS2 truncated mutant ACS2Δ[1] (Figs. 1a, 3a). Within the Lys[438]–Ser[460] region, there are four putative TMP-mediated cleavage sites (Fig. 3c), which generate the P4 and P5 peptides. Because these two peptides were detected at a relatively low quantity in comparison with P1, P2, and P3, they may be further degraded by TMP during proteolysis. Considering the existence of P5 together with the different proteolytic behaviors of Del2-GST and Del3-GST by TMP (Fig. 4a, b), it is highly possible that the carboxyl side of Lys[438] may be the cleavage site most distal to the C-terminus of the full-length protein. Further cleavages by TMP towards the N-terminal region of LeACS2 seem to be inhibited by the α-helix domain (*H14*; Huai *et al.* 2001). Correspondingly, the cleavage at Lys[438] and Glu[447] (or Lys[448]) may produce a 49- and 50-kDa LeACS2 isoform, respectively. Furthermore, the molecular weights of *in vitro*-processed LeACS2 isoforms appeared to correlate well with those of *in vivo*-truncated LeACS2 isoforms detected using the SDS-boiling method (Fig. 3a), which suggested the likelihood that TMP may be involved in the *in vivo* proteolysis of LeACS2. If this is the case, this proteolytic modification may remove the Ser[460] phosphorylation site from the C-terminus of LeACS2 and may eliminate the influence of phosphorylation/dephosphorylation on the stability of this enzyme.

We constructed a series of nested C-terminal deletion mutants of LeACS2 to determine whether the TMP cleavage sites are, indeed, located at the N-terminal side of the Ser[460] phosphorylation site. The data shown in Fig. 4b unequivocally demonstrates that these cleavages occur on the N-terminal side of the Ser[460] residue. In the case of the Del2-GST mutant, the proteolytic efficiency of TMP was severely reduced, probably because the EKSGDK region is flanked by an α-helix (*H14*; Fig. 4a) and a compact GST tag. These

structural constraints are likely to have blocked the protease from accessing its substrate sites.

In summery, the TMP isolated in the present study processes the C-terminus of tomato ACS (LeACS2) *in vitro*. A similar processing pattern was observed for *in vivo* proteolysis, suggesting that this protease may be the candidate responsible for the *in vivo* C-terminal truncation of ACS in tomato fruits. Because the truncated forms of LeACS2 enzyme are active *in vitro*, the *in vitro* limited proteolysis of LeACS2 by this protease suggests a model for *in vivo* proteolytic modification of ACS. However, this hypothesis awaits further *in vivo* evidence, such as RNAi or loss-of-function mutations, to support the function of this protease in fruit.

References

Alexander L, Grierson D (2002). Ethylene biosynthesis and action in tomato: A model for climacteric fruit ripening. *J Exp Bot* **53**, 2039–2055.

Bleecker AB, Kende H (2000). Ethylene: A gaseous signal molecule in plants. *Annu Rev Cell Dev Biol* **16**, 1–18.

Chae HS, Faure F, Kieber JJ (2003). The *eto-1*, *eto-2*, and *eto-3* mutations and cytokinin treatment increase ethylene biosynthesis in *Arabidopsis* by increasing the stability of ACS protein. *Plant Cell* **15**, 545–559.

Edelman L, Kende H (1990). A comparison of 1-aminocyclopropane-1-carboxylic acid synthase *in vitro* translation product and *in vivo*-labeled protein in ripening tomatoes. *Planta* **182**, 635–638.

Edman P (1950). Method for determination of the amino acid sequence in peptides. *Acta Chem Scand* **4**, 283.

Felix G, Grosskopf DG, Regenass M, Basse CW, Boller T (1991). Elicitor-induced ethylene biosynthesis in tomato cells: Characterization and use as a bioassay for elicitor action. *Plant Physiol* **97**, 19–25.

Felix G, Regenass M, Spanu P, Boller T (1994). The protein

phosphatase inhibitor calyculin A mimics elicitor action in plant cells and induces rapid hyperphosphorylation of specific proteins as revealed by pulse-labeling with [^{33}P] phosphate. *Proc Natl Acad Sci USA* **91**, 952–956.

Fluhr R, Mattoo AK (1996). Ethylene: Biosynthesis and perception. *Crit Rev Plant Sci* **15**, 479–523.

Gane R (1934). Production of ethylene by some ripening fruits. *Nature* **134**, 1008.

Ge L, Liu JZ, Wong WS *et al.* (2000). Identification of a novel multiple environmental factor responsive 1-aminocyclopropane-1-carboxylate synthase gene, NT-ACS2, from tobacco. *Plant Cell Environ* **23**, 1169–1182.

Hirel PH, Schmitter JM, Dessen P, Fayat G, Blanquet S (1989). Extent of N-terminal methionine excision from *Escherichia coli* proteins is governed by the side-chain length of the penultimate amino acid. *Proc Natl Acad Sci USA* **86**, 8247–8251.

Huai Q, Xia YH, Chen YQ, Callahan B, Li N, Ke HM (2001). Crystal structures of 1-aminocyclopropane-1-carboxylate (ACC) synthase in complex with aminoethoxyvinylglycine and pyridoxal-5'-phosphate provide new insight into catalytic mechanisms. *J Biol Chem* **276**, 38 210–38 216.

Janzik I, Macheroux P, Amrhein N, Schaller A (2000). *LeSBT1*, a subtilase from tomato plants. *J Biol Chem* **275**, 5193–5199.

Kende H (1993). Ethylene biosynthesis. *Annu Rev Plant Physiol Plant Mol Biol* **44**, 283–307.

Kim CY, Liu YD, Thorne ET *et al.* (2003). Activation of a stress-responsive mitogen-activated protein kinase cascade induces the biosynthesis of ethylene in plants. *Plant Cell* **15**, 2707–2718.

Kim WT, Yang SF (1992). Turnover of ACC synthase protein in wounded tomato fruit tissue. *Plant Physiol* **100**, 1126–1131.

Klee HJ (2002). Control of ethylene-mediated processes in tomato at the level of receptors. *J Exp Bot* **53**, 2057–2063.

Li JF, Qu LH, Li N (2005). Isolation of a tomato protease that may be involved in proteolysis of 1-aminocyclopropane-1-carboxylate synthase. *J Integrat Plant Biol* **47**, 1220–1227.

Li N, Huxtable S, Yang SF, Kung SD (1996). Effects of N-terminal deletions on 1-aminocyclopropane-1-carboxylate synthase activity. *FEBS Lett* **378**, 286–290.

Li N, Mattoo AK (1994). Deletion of the carboxyl-terminal region of 1-aminocyclopropane-1-carboxylic acid synthase, a key protein in the biosynthesis of ethylene, results in catalytically hyperactive, monomeric enzyme. *J Biol Chem* **269**, 6908–6917.

Li N, Parsons BL, Liu D, Mattoo AK (1992). Accumulation of wound-inducible ACC synthase transcript in tomato fruit is inhibited by salicylic acid and polyamines. *Plant Mol Biol* **18**, 477–487.

Lizada MCC, Yang SF (1979). A simple and sensitive assay for 1-aminocyclopropane-1-carboxylic acid. *Anal Biochem* **100**, 140–145.

Nakajima N, Nakagawa N, Imaseki H (1988). Immunochemical difference of wound-induced 1-aminocyclopropane-1-carboxylate synthase from the auxin-induced enzyme. *Plant Cell Physiol* **29**, 989–998.

Nakajima N, Mori H, Yamazaki K, Imaseki H (1990). Molecular cloning and sequence of a complementary DNA encoding 1-aminocyclopropane-1-carboxylate synthase induced by tissue wounding. *Plant Cell Physiol* **31**, 1021–1029.

Nakatsuka A, Murachi S, Okunishi H *et al.* (1998). Differential expression and internal feedback regulation of 1-aminocyclopropane-1-carboxylate synthase, ACC oxidase, and ethylene receptor genes in tomato fruit during development and ripening. *Plant Physiol* **118**, 1295–1305.

Oeller PW, Wong LM, Taylor LP, Pike DA, Theologis A (1991). Reversible inhibition of tomato fruit senescence by antisense 1-aminocyclopropane-1-carboxylate synthase RNA. *Science* **254**, 437–439.

Rottmann WH, Peter GF, Oeller PW *et al.* (1991). 1-Aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and floral senescence. *J Mol Biol* **222**, 937–961.

Sato T, Theologis A (1989). Cloning the mRNA encoding 1-aminocyclopropane-1-carboxylate synthase, the key enzyme for ethylene biosynthesis in plants. *Proc Natl Acad Sci USA* **86**, 6621–6625.

Sato T, Oeller PW, Theologis A (1991). The 1-aminocyclopropane-1-carboxylate synthase of cucurbita: Purification, properties, expression in *Escherichia coli*, and primary structure determination by DNA sequence analysis. *J Biol Chem* **266**, 3752–3759.

Jian-Feng LI *et al.*: Cleavage of the Carboxyl-Terminus of LEACS2, a Tomato 1-Aminocyclopropane-1-Carboxylic Acid
Synthase Isomer, by a 64-kDa Tomato Metalloprotease Produces a Truncated but Active Enzyme

1363

Satoh S, Esashi Y (1986). Inactivation of 1-aminocyclopropane-1-carboxylic acid synthase of etiolated mung bean hypocotyl segments by its substrate, *S*-adenosylmethionine. *Plant Cell Physiol* **91**, 1036–1039.

Sebastia CH, Hardin SC, Clouse SD, Kieber JJ, Huber SC (2004). Identification of a new motif for CDPK phosphorylation *in vitro* that suggests ACC synthase may be a CDPK substrate. *Arch Biochem Biophys* **428**, 81–91.

Spanu P, Felix G, Boller T (1990). Inactivation of stress-induced 1-aminocyclopropane-1-carboxylate synthase *in vivo* differs from substrate-dependent inactivation *in vitro*. *Plant Physiol* **93**, 1482–1485.

Spanu P, Grosskopf DG, Felix G, Boller T (1994). The apparent turnover of 1-aminocyclopropane-1-carboxylate synthase in tomato cells is regulated by protein phosphorylation and dephosphorylation. *Plant Physiol* **106**, 529–535.

Tatsuki M, Mori H (2001). Phosphorylation of tomato 1-aminocyclopropane-1-carboxylic acid synthase, LE-ACS2, at the C-terminal region. *J Biol Chem* **276**, 28051–28057.

Tsuchisaka A, Theologis A (2004). Heterodimeric interactions among the 1-aminocyclopropane-1-carboxylate synthase polypeptides encoded by the *Arabidopsis* gene family. *Proc Natl Acad Sci USA* **101**, 2275–2280.

van der Straeten D, van Wiemeersch L, Goodman HM, van Montagu MV (1990). Clone and sequence of two different cDNAs encoding 1-aminocyclopropane-1-carboxylate synthase in tomato. *Proc Natl Acad Sci USA* **87**, 4859–4863.

Wang KLC, Yoshida H, Lurin C, Ecker JR (2004). Regulation of ethylene gas biosynthesis by the *Arabidopsis* ETO-1 protein. *Nature* **428**, 945–950.

(Managing editor: Wei WANG)